

# Opportunities to Germinate and Grow of *Alicyclobacillus acidoterrestris* Spores in the Presence of Organic Acids

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## Abstract

*Alicyclobacillus acidoterrestris* is an acidophilic, spore-forming bacterium that may cause spoilage of pasteurized fruit juices and beverages, producing compounds associated with disinfectant-like odour (guaicol, halophenols).

The aim of this study was to determine the effectiveness of chosen organic acids against spores of five *Alicyclobacillus acidoterrestris* strains isolated from Polish concentrated apple juices as well as to mark MICs of these acids.

Minimum inhibitory concentrations (MICs) of chosen organic acids and their salts were determined in BAT-broth (pH 4.0) up to seven days at 45°C. At this set of conditions the hierarchy of effectiveness for spores inactivation was as follows: sodium benzoate (MIC=0.1 g/L) > potassium sorbate (MIC=0.25 g/L) > acetic acid (MIC=0.7 g/L) > propionic acid (MIC=0.7-1.0 g/L) > lactic acid (MIC=3.0-4.0 g/L) > adipic acid (MIC=6.0-8.0 g/L) > succinic acid (MIC=2.0-20.0 g/L) > malic acid (40.0-60.0 g/L) > tartaric acid (MIC>50.0 g/L) > citric acid (MIC=50.0g/L-70.0 g/L).

The non-germinated spores were removed from the acidic environment by membrane filtration (Milipore Corp.) and incubated on BAT-agar. The viability of spores was varied and depended on the used acid. The high degree of spore inactivation was achieved - only 2.0-6.0% and 3.0-4.4% spores were capable of germination and further growth after removing from lactic acid and potassium sorbate environment respectively. However, 80.0-92.0% spores collected on filters showed normal germination after removing from adipic acid environment. In this case acid-induced inhibition was almost totally reversible.

## Keywords

Organic Acids; MIC; *Alicyclobacillus Acidoterrestris*

## Introduction

The extreme resistance of bacterial endospores to physical and chemical treatments makes them a significant problem for the food industry. One of these

spore-forming is an acidophilic bacterium, *Alicyclobacillus acidoterrestris*, which may cause spoilage of pasteurized fruit juices and beverages. Spoilage is manifested as the formation of off-flavours and odours from compounds such as guaicol and halophenols. *A. acidoterrestris* strains show the ability to germinate and grow at the pH range from 2.0 to 6.0 at temperature 23 - 55°C, with optimum range 42-53°C.

*A. acidoterrestris* has been isolated from a number of different juices, including apple, orange, pear, cherry, grapefruit, mango, tomato, white grape, aloe vera, pineapple, lemon and passion fruit. These bacteria were also isolated from various types of acidic beverages, including Ice tea.



FIG. 1 SCANNING ELECTRON PHOTOMICROGRAPH OF THE SPORE-FORMING *ALICYCLOBACILLUS ACIDOTERRESTRIS* TO-29/04/02 STRAIN, EXTENSION 28 000 X

Organic acids are compounds contain naturally in fruits, and are known to have antimicrobial activities. The antimicrobial action of organic acids is primarily based on their ability to reduce the pH. Weak lipophilic organic acids (acetic, propionic, sorbic, benzoic) penetrate the cell membrane in the

undissociated form to inhibit growth or cause bactericidal action by dissociating and acidifying the cytoplasm. Mechanisms of antimicrobial activity are based on inhibition of enzymes, membrane function, nutrient transport and overall metabolic activity. The inhibitory effect of organic acids is caused by their undissociated form ( $pK_a$ ).

Fruits and fruit juices naturally contain organic acids as citric, malic and tartaric acids. These are characterized by a lower  $pK_a$  than those of acetic and lactic acids (Tab. 2). The lower antimicrobial activity of citric and malic acid compared with lactic and acetic acids at the same pH means that fruits and their juices may allow survival and growth of microorganisms despite their low pH. The pH of fruit juices ranges from 2.9 to 4.3 depending on fruit variety.

Content of citric, malic and tartaric acid in commercial fruit juices is regulated by the Code of Practice AIJN (Tab. 1). Additionally small amounts of acids: succinic, oxalic, formic, acetic, lactic, salicylic and benzoic appear in fruits.

TABLE 1 ORGANIC ACIDS CONTENT IN FRUIT JUICES  
ACCORDING TO CODE OF PRACTICE AIJN

Kind of Juice	Minimum Soluble Solid [°Bx]	Citric Acid Content [g/L]	L(-) Malic Acid Content [g/L]	L(+) Tartaric Acid Content [g/L]
Black currant juice	11.6	26.0 – 42.0	1.0 – 4.0	-
Lemon juice	8.0	45.0 – 63.0	1.0 – 7.5	-
Grapefruit juice	10.0	8.0 – 20.0	0.2 – 1.2	-
Apple juice	11.2	0.05 – 0.15	min. 3.0	
Raspberry juice	7.0	9.0 – 22.0	0.2 – 1.2	
Orange juice	11.2	6.3 – 17.0	0.8 – 3.0	-
Tomato juice	5.0	2.0 – 5.0	0.1 – 0.6	-
Strawberry juice	7.0	5.0 – 11.0	0.6 – 5.0	-

The relative effectiveness of different compounds has often been expressed in terms of their minimum inhibitory concentrations (MICs). The MIC is the lowest concentration of an antimicrobial that inhibits the visible growth of an organism under a set of conditions.

The aim of this study was to determine the effectiveness of chosen organic acids against spores of

five *Alicyclobacillus acidoterrestris* strains isolated from Polish concentrated apple juices as well as to mark MICs of these acids.

## Materials and Method

### Tested Organisms

Five *A. acidoterrestris* strains (TO-29/4/02, TO-117/02, TO-224/1/05, TO-169/06 and U-44/25/06) used in this study were isolated from Polish concentrated apple juices. The International Federation of Fruit Juice Producers method was used to detect these *Alicyclobacillus* strains. Confirmation of *A. acidoterrestris* was based on the utilization of erythritol, with acid production and guaiacol production in YSG medium with vanillic acid.

### Spore Production

Spores were produced based on the method described in reference, with some modifications. A fresh culture suspension of *A. acidoterrestris* in BAT-broth was spread-plated onto ten potato dextrose agar plates pH 4.0 (Oxoid). The inoculated plates, packed in sealable plastic bags to prevent drying, were incubated at 45°C for ca. 10 days, until over 90% spores had developed. The degree of sporulation was monitored by microscopy after staining with malachite green. The sporulated agar plates were harvested by flooding with 2.5 mL of sterile, deionised water and scraping with a spreading loop. The spores were washed by centrifugation three times (17 000 x g for 10 min at 4°C). Finally the spore pellets were re-suspended in 5 mL of sterile deionised water and stored at 4°C until use. The number of spores was evaluated after serial dilutions and subsequent incubation on BAT-agar (Merck) for 5 days at 45°C.

### Media

BAT-broth was supplemented by organic acid and final pH was adjusted to 4.0 with 1M NaOH, 2.5 M NaOH or 0.5 M H<sub>2</sub>SO<sub>4</sub>.

Malic acid (Fluka, >99% purum) was used at a concentration range from 1.0 to 60.0 g/L. Citric acid (POCH p.a. 99,8%) was used at the same concentrations and additionally at 70.0 g/L. The applied concentrations of tartaric acid (Merck p.a. 99,5%) were from 1.0 to 50.0 g/L. Succinic acid (Fluka, >99% purum p.a.) was used at a concentration range from 1.0 to 20.0 g/L. Concentration of adipic acid (Aldrich, 99% p.a.) was 2.0-8.0 g/L. Lactic acid (POCH 80% p.a.) was added at a concentration 1.0-4.0

g/L Propionic (Riedel-de Haën, puriss >99%) and acetic (AppliChem, p.a. 99.81%) acids were used at a concentrations 0.5-1.0 g/L. Sodium benzoate (POCh min. 99,5% p.a.) and potassium sorbate (POCh min. 99%) were added at final concentration 0.05-1.0 g/L. Chosen properties of organic acid are listed in Table 2.

TABLE 2 CHOSEN PROPERTIES OF ORGANIC ACID ADDED TO BAT-BROTH

Common Acid Name	Molecular Formula	Molecular Weight	pK <sub>a</sub>	Stock Solution
Acetic	C <sub>2</sub> H <sub>4</sub> O <sub>2</sub>	60.06	4.75	10% in water
Adipic	C <sub>6</sub> H <sub>10</sub> O <sub>4</sub>	146.14	4.43	5% in water and ethanol (1:1)
Benzoic	C <sub>7</sub> H <sub>6</sub> O <sub>2</sub>	122.12	4.19	10% in water
Citric	C <sub>6</sub> H <sub>8</sub> O <sub>7</sub>	192.14	3.14	20 or 10% in water
Lactic	C <sub>3</sub> H <sub>6</sub> O <sub>3</sub>	90.08	3.86	10% in water
Malic	C <sub>4</sub> H <sub>6</sub> O <sub>5</sub>	134.09	3.40	20 or 10% in water
Propionic	C <sub>3</sub> H <sub>6</sub> O <sub>2</sub>	74.09	4.87	10% in water
Sorbic	C <sub>6</sub> H <sub>8</sub> O <sub>2</sub>	112.14	4.76	10% in water
Succinic	C <sub>4</sub> H <sub>6</sub> O <sub>4</sub>	118.09	4.16	5% in water
Tartaric	C <sub>4</sub> H <sub>6</sub> O <sub>6</sub>	150.09	2.98	10% in water

#### Determination Minimum Inhibitory Concentration (MIC)

In this study minimum inhibitory concentration was the lowest concentration of an acid that will prevent the growth of *A. acidoterrestris* spores under a set of conditions. MICs for each of ten acids for five *A. acidoterrestris* strains were determined in BAT-broth at pH 4.0. Before incubation (at 45°C) samples were inoculated (100 spores/mL) and subjected to heat shock (80°C ± 1°C, 10 min). Turbidity was measured after 3, 5 and 7 days with a HACH 2100N turbidimeter. Viability was checked by sub-culturing onto BAT-agar. No acids control was performed. The assays were performed using at least two independent samples.

#### Recovery

Cultures in which, after 7 days of incubation, no growth was observed, were filtered (cellulose filters Millipore Corp. 0,45 µm) to separate non-germinated

spores. Filters were incubated on the BAT-agar (Merck) at 45°C for 5 days. Based on the number of colonies the recovery was calculated as a % of inoculum.

## Results

### Characteristics of *Alicyclobacillus acidoterrestris* Strains

*A. acidoterrestris* strains isolated from Polish concentrated apple juices and used in this study were previously characterized (Tab. 3). They can growth in a temperature range from 20 to 55°C with an optimum between 37 and 50°C and a pH from 2.5 to 6.0 with the optimum between 3.5 and 5.0. Spores of these *A. acidoterrestris* strains were also heat resistant. D<sub>95</sub> value were 3.8 to 15.1 min in commercial apple juice (11.2°Bx, pH 3.4) and from 8.2 to 29.8 min in concentrated apple juice (70.0°Bx, pH 3.12).

### Germination and Growth of *Alicyclobacillus acidoterrestris* Spores in the Presence of Organic Acids

The effect of organic acids on the growth of *A. acidoterrestris* are presented as MICs in Table 4 and 4a. Malic, citric and tartaric acid were very well tolerated by tested spores. All tested *A. acidoterrestris* spores germinated and grew in BAT-broth up to 30 g/L L(-) malic acid and citric acid content. In case when L(+) tartaric acid was added to BAT-broth growth was observed even up to 50.0 g/L. In most of fruit juices there is no high content of acids observed (Tab. 1). At these concentrations of malic, citric and tartaric acids growth of *A. acidoterrestris* was higher than in no acid control samples. This indicates that these acids were utilized as a nutrient (data not presented).

MICs of L(-) malic acid were 40.0-60.0 g/L depending on the strains (Tab. 4). In these concentrations *A. acidoterrestris* spores were not totally destroyed. 8.0-15.0% spores were capable of germination when they were removed from acidic environment by membrane filtration.

Citric acid MICs for tested spores were 40.0 to 70.0 g/L. 2.0 to 20.0% spores retained their capability to germinate and grow.

Varied sensitivity of *A. acidoterrestris* spores on succinic acid in the BAT-broth was observed. MICs range were very wide – from 2.0 even up to 20.0 g/L (Tab. 4). Also in this case, spores were not completely destroyed. 10.0 to 15.3% of them survived in this condition and were capable of germination and further growth.

TABLE 3 CHARACTERISTICS OF ALICYCLOBACILLUS ACIDOTERRESTRIS STRAINS USING IN THIS STUDY

<i>A. acidoterrestris</i> Strains	Growth Temperature Range [°C]	Optimum Temperature [°C]	Growth pH Range	Optimum pH	D <sub>95</sub> in Apple Juice: 11.2°Bx pH 3.40 [min]	D <sub>95</sub> in Concentrated Apple Juice: 70.0°Bx pH 3.12 [min]
TO-29/04/02	20.0-55.0	50.0	3.0-6.0	3.5-4.5	10.7	17.9
TO-117/02	20.0-55.0	37.0-55.0	3.0-6.0	4.0-4.5	8.0	15.4
TO-224/1/05	20.0-55.0	45.0	2.5-6.0	4.0-5.0	-	-
U-44/25/06	20.0-55.0	45.0	3.0-6.0	3.5-5.0	15.1	29.8
TO-169/06	20.0-55.0	45.0-50.0	3.0-6.0	4.0-5.0	3.8	8.2

TABLE 4 MIC VALUES OF ORGANIC ACIDS AGAINST ALICYCLOBACILLUS ACIDOTERRESTRIS SPORES IN BAT-BROTH (pH 4.0)

<i>A. acidoterrestris</i> Strains	L(-) Malic Acid		Citric Acid		L(+) Tartaric Acid**	Succinic Acid	
	MIC [g/L]	Recovery* [%]	MIC [g/L]	Recovery* [%]	MIC [g/L]	MIC [g/L]	Recovery* [%]
TO-29/04/02	40.0	10.0	60.0	12.0	> 50.0	12.0	10.0
TO-117/02	50.0	9.5	70.0	2.0	> 50.0	6.0	12.6
TO-224/1/05	40.0	10.3	40.0	15.0	> 50.0	2.0	15.0
U-44/25/06	60.0	15.0	50.0	11.5	> 50.0	6.0	12.0
TO-169/06	60.0	8.0	70.0	20.0	> 50.0	20.0	15.3

\*Recovery – number the spores capable to grow, after removing from acidic medium, as a % of inoculum

\*\*Further increasing the content of tartaric acid was not possible due to the precipitation of insoluble tartrate in the medium

TABLE 4a MIC VALUES OF ORGANIC ACIDS AGAINST ALICYCLOBACILLUS ACIDOTERRESTRIS SPORES IN BAT-BROTH (pH 4.0)

<i>A. acidoterrestris</i> Strains	Adipic Acid		Lactic Acid		Acetic Acid		Propionic Acid	
	MIC [g/L]	Recovery* [%]	MIC [g/L]	Recovery* [%]	MIC [g/L]	Recovery* [%]	MIC [g/L]	Recovery* [%]
TO-29/04/02	8.0	80.0	4.0	2.0	0.7	8.0	1.0	15.0
TO-117/02	6.0	92.0	3.0	6.0	0.7	7.0	1.0	9.0
TO-224/1/05	8.0	85.0	3.0	3.0	0.7	20.0	0.7	22.0
U-44/25/06	8.0	90.0	4.0	5.0	0.7	11.0	1.0	9.0
TO-169/06	8.0	90.0	4.0	5.0	0.7	30.0	1.0	30.0

\*Recovery – number the spores capable to grow, after removing from acidic medium, as a % of inoculum

For adipic acid in the concentration range of 1.0 to 4.0 g/L growth of all tested of *A. acidoterrestris* strains was observed. MICs of this acid for the most strains was 8.0 g/L, but up to 80.0-92.0% spores were able to germinate after removing from medium (Tab. 4a).

Lactic acid MICs were 3.0-4.0 g/L in this study and what is interesting, most of the spores were inactivated.

Only 2.0-6.0% spores were capable of germination and further growth.

Propionic and acetic acids prevented the germination and growth of *A. acidoterrestris* spores. MICs of these acids was 0.7-1.0 and 0.7 g/L respectively. However, after removing from acid environmental the recoveries of the spores were up to 30.0% (Tab. 4a).

TABLE 5 MIC VALUES OF PRESERVATIVES (ORGANIC ACIDS SALT) AGAINST ALICYCLOBACILLUS ACIDOTERRESTRIS SPORES IN BAT-BROTH (pH 4.0)

<i>A. acidoterrestris</i> Strains	Sodium Benzoate		Potassium Sorbate	
	MIC [g/L]	Recovery* [%]	MIC [g/L]	Recovery* [%]
TO-29/4/02	0.1	8.2	0.25	4.2
TO-117/02	0.1	12.0	0.25	3.5
TO-224/1/05	0.05	8.8	0.25	3.0
U-44/25/06	0.1	15.6	0.25	4.0
TO-169/06	0.1	21.0	0.25	4.4

\*Recovery – number the spores capable to grow, after removing from acidic medium, as a % of inoculum

For the majority of *A. acidoterrestris* spores MIC of sodium benzoate was 0.1 g/L. MIC of potassium sorbate was the same for all tested strains – 0.25 g/L. If sodium benzoate was used 8.2-21.0% spores were able to germinate and grow after removing from medium. Potassium sorbate was more effective – recovered only 3.0 – 4.4% spores (Tab. 5).

MICs of malic, citric and tartaric acids for vegetative cells of *A. acidoterrestris* (VF strain isolated in USA from apple juice) obtained in Hsiao and Siebert, 1999, works, are several times lower than those obtained in our study for spores. MICs determined for the citric and malic acids was only 4.58 g/L and 7.07 g/L for tartaric acid. It is possible that at higher concentrations of acid, bacteria had not managed to grow during 2 days of incubation.

However, lactic acid MIC for vegetative cells (5.39 g/L) was higher than of in our trial for spores. MICs values of acetic acid and sodium benzoate for *A. acidoterrestris* spores in our study were similar to reported in other works. Tokuda, 2007, reported that citric acid and malic acid did not show a significant inhibition of growth *A. acidocaldarius* AC-1 (strain isolated from a fruit juice drink in Japan), however, the other acids were inhibitory especially at lower pH. The decreasing order of inhibition was acetic acid, adipic acid, lactic acid, fumaric acid. Acetic acid was found to strongly inhibit growth. Sodium benzoate and potassium sorbate were successfully used to inhibition of *A. acidoterrestris* growth in apple juice by Walker and Philips, 2008. In apple juice, 0.1 g/L sodium benzoate or potassium sorbate inhibited growth of  $10^1$  cells/mL *A. acidoterrestris* while 0.5 g/L inhibited growth  $10^4$  cells/mL during 29 days incubation at 30°C.

## Conclusions

Obtained results showed that hierarchy of effectiveness for *A. acidoterrestris* spores inactivation is as follows: sodium benzoate > potassium sorbate > acetic acid > propionic acid > lactic acid > adipic acid > succinic acid > malic acid > tartaric acid > citric acid. In some cases acid-induced inhibition was almost totally reversible (adipic acid) but a high degree of spore inactivation can also be achieved (lactic acid, potassium sorbate). Registered in our work hierarchy of effectiveness some organic acids correlated with values of their dissociation constant. We can easily observe that the acids with higher dissociation constant have a larger inhibitory effect.

The study confirmed the acidophilic character of *Alicyclobacillus acidoterrestris* bacteria. The results can be used in soft drink industry where chemical preservatives such as potassium sorbate and sodium benzoate may be used to control *A. acidoterrestris* spores germination.

The lactic acid may be used as a substitute of citric acid in the production of nectars and beverages due to the high degree of *A. acidoterrestris* spores inactivation, but further research on shelf life in this type of products are needed.

An interesting approach for future researches could be the study on using same acids as additives to water for fruits washing to reduce the initial spore number.

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She is an author of many articles, apart from those mentioned in references: „The combined effect of high pressure and nisin or lysosyme on the inactivation *Alicyclobacillus acidoterrestris* spores in apple juice”, High Pressure Research, 32, 119-127, 2012, „Formation of guaiacol from vanillic acid by *Alicyclobacillus* sp. – a model study in apple juice”, Pr. Inst. Lab. Bad. Przem. Spoż., 65, 39-46, 2010 (in Polish), „Inactivation of *Alicyclobacillus acidoterrestris* spores by chlorine dioxide in model suspensions”, Przem. Ferm. Owoc. Warz., 3, 12-16, 2012 (in Polish).

She got interested in microbiological quality of fruit juices in 2001, especially in the detection of *Alicyclobacillus acidoterrestris* which has become the subject of her PhD thesis.

Dr. Sokołowska is member of National Standards Body in Poland, (Technical Committee No 3 for Food Microbiology) and Technical Committee for Agricultural and Food in Polish Center of Accreditation.